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Induction of Spermatogenesis in Male Japanese Eel, *Anguilla japonica*, by a Single Injection of Human Chorionic Gonadotropin

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ABSTRACT—Cultivated males of the Japanese eel (*Anguilla japonica*) were given a single injection of human chorionic gonadotropin (HCG; 5 IU/g BW), and histological changes in the testis were observed. Profiles of serum androgens (11-ketotestosterone and testosterone) and production of androgens by the testis *in vitro* were also measured. 1) *Histology of the testis*: Prior to HCG treatment, germ cells of male Japanese eel were all spermatogonia, and the morphology of Sertoli cells and Leydig cells indicated little activity. One day after injection, the first effect of HCG treatment was observed, consisting of the activation of Leydig and Sertoli cells. This was followed by proliferation of spermatogonia, which began after three days. After twelve days, some germ cells had begun meiosis. Spermatozoa were first observed after eighteen days. 2) *Serum androgen profiles*: Serum androgen levels were relatively low before HCG treatment, but had increased dramatically by one day after the treatment, and thereafter high levels were maintained throughout spermatogenesis. 3) *In vitro production of androgens by the testis*: The testis of uninjected eels produced androgens, principally 11-ketotestosterone, when cultured *in vitro* with HCG. Production was proportional to the concentration of HCG. These results indicate that a single injection of HCG can induce the proliferation of spermatogonia, the initiation of meiosis, and the induction of spermatogenesis. The phenomenon in the eel is associated with remarkable endocrinological changes, including the development of Leydig and Sertoli cells, and an increase in androgen production.

INTRODUCTION

In male vertebrates, primordial germ cells in the testis undergo spermatogenesis to form spermatozoa which, after passing through a process of final maturation, have the ability to fertilize ova. In teleosts, most recent endocrinological studies of spermatogenesis have concentrated on late spermatogenesis and spermiation (see [1]). However, the mechanisms of early spermatogenesis, including the proliferation of spermatogonia and the beginning of meiosis, are not clear.

Under conditions of cultivation, male Japanese eel (*Anguilla japonica*) have immature testes containing spermatogonia, but no later developmental stages of germ cells. However, if these eels are injected with exogenous gonadotropin, their testes resume spermatogenesis, and germ cells undergo meiosis to become spermatozoa [2]. This suggests that the cultivated male Japanese eel may be a good system to analyze the mechanisms controlling the process of spermatogenesis.

Androgens are produced by the testis in response to gonadotropin (see [3]), and have been implicated in the process of spermatogenesis [4]. Testosterone is the major androgen of higher vertebrates [4]. 11-Ketotestosterone was first identified by Idler *et al.* [5] as a major androgenic steroid in male sockeye salmon (*Oncorhynchus nerka*), and since then has been shown to be present in the plasma of a large variety of male teleosts (see [6]). Increased production of these

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androgens has been measured during teleost spermatogenesis [6], however, their function remains unclear.

The present study analyzed morphological changes of germinal and somatic elements in the testis and change of two serum androgen (11-ketotestosterone and testosterone) levels following induction of spermatogenesis by HCG treatment in cultivated eel. In addition, *in vitro* studies were carried out to examine whether the eel testis could produce the two androgens in response to HCG.

MATERIALS AND METHODS

Animals

Fifteen males of the cultivated Japanese eel (180–200 g in body weight) were purchased from a commercial eel supplier in October. They were kept in circulating freshwater tanks with a capacity of 500 l at 20°C. Fish were not fed throughout the experimental period. All fish were marked to distinguishi each individual for serial samplings.

For seven eels, a single injection of human chorionic gonadotropin (HCG) dissolved in saline (150 mM NaCl) was given intramuscularly at a dose of 5 IU per g body weight. Five control fish were injected only with saline. Each fish was sampled before injection of either HCG and saline, and 1, 3, 6, 12 and 18 days postinjection. The fish were anesthetized with 0.1% ethyl aminobenzoate, and a few small fragments of testis were taken by biopsy. 0.5 ml of blood was also collected from the caudal vasculature by syringe. Serum was immediately separated at 4°C by centrifugation at 1500 g and stored at −80°C until use.

Morphological studies

The fragments of testis were fixed in a 2% paraformaldehyde—2% glutaraldehyde mixture in 0.1 M cacodylate buffer at pH 7.4, postfixed in 1% osmium tetroxide in the same buffer, and embedded in epoxy resin according to standard procedures. Section, 1 μm, thick were stained with toluidine blue for light microscopic examination. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a JEOL 100-CX transmission electron microscope operated at 80 kV.

Two kinds of quantitative analyses were carried out by light and electron microscope observations. First, 5 random sections from each of 5 testis fragments originating from 12 eels were examined, and the number of cysts containing each germ cell type was counted. The results were expressed in terms of percent of cysts of a particular germ cell type per total cysts observed. The cysts of the following 5 germ cell types were distinguished and counted: 1) type A psermatogonia and early type B spermatogonia, 2) late type B spermatogonia, 3) primary and secondary spermatocytes, 4) spermatids, and 5) spermatozoa. Isolated type A spermatogonia or groups of two cells surrounded by Sertoli cells, were counted as cysts. Second, the area of nuclei in Sertoli cells and the area of mitochondria in Leydig cells were measured on electron micrographs by using an interactive image analysis system IBAS-I (KONTRON, München, West Germany). These parameters were used as indicators of the growth and development of each cell type.

In addition, 18 days after HCG injection, the eels were sacrificed and testis fragments were fixed in Bouin's solution, and embedded in paraffin according to standard procedures. Serial 5 μm sections were stained with Masson's trichrome staining. The number of spermatocytes in each of 31 cysts was counted by IBAS-I and II.

Synthesis of androgen in immature testis

Immature testes of 3 uninjected fish were carefully removed and transferred to glass petri dishes containing physiological saline solution for eel (NaCl 150 mM, KCl 3.0 mM, $MgCl_2$ 3.5 mM, $CaCl_2$ 5.0 mM, Hepes 10 mM at pH 7.5 by 0.1 N NaOH). Testes were minced with scissors, and 50 mg of testicular fragments were incubated in plastic tissue-culture dishes (Costar, U.S.A.) containing 1 ml physiological saline in the presence or absence of various concentrations (0.01 to 10 IU/ml) of HCG for 18 hr at 20°C. At the end of incubation, the incubation medium was collected and stored at −20°C until assayed for 11-ketotestosterone (11-oxotestosterone) and testosterone. 11-Ketotestosterone and testosterone of serum and incubation medium were measured by

specific radioimmunoassay according to the method of Ueda *et al.* [7].

Results were expressed as means and standard errors (SEM). Control and experimental groups did not differ in any measured characteristic prior to injection with saline or HCG (Student's t-test, $P < 0.05$). Changes over time were measured by two-way ANOVA, and differences in means within each group were measured by paired t-tests.

RESULTS

Morphological changes in the testis after HCG injection

1. *Germ cells*

Before HCG injection, all germ cells present in the testis of male eels were type A spermatogonia or early type B spermatogonia (Fig. 1a). Both

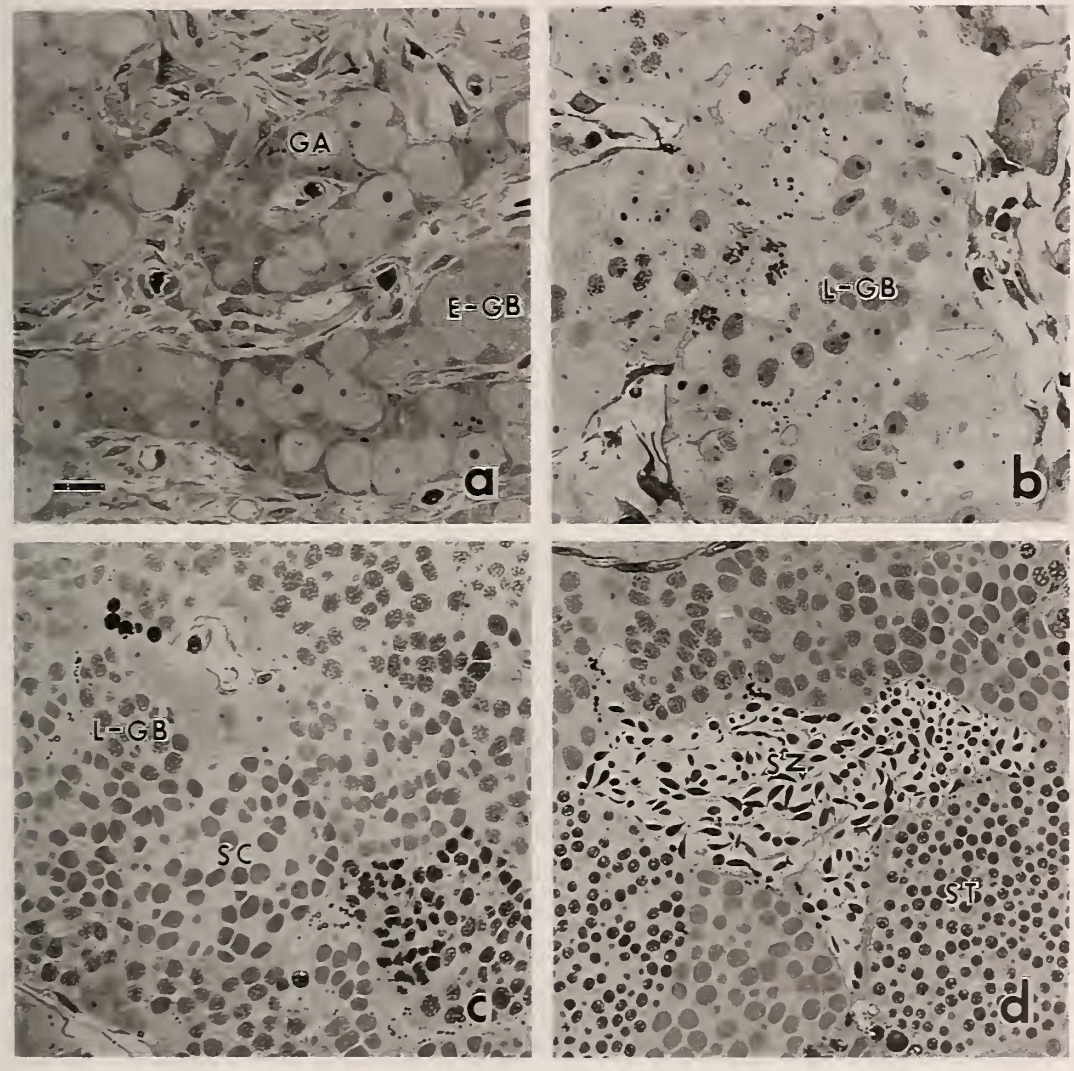

FIG. 1. Photomicrographs of the testis of cultivated Japanese eel injected with HCG. a) Portion of the testis before HCG injection. b) Portion of the testis 3 days after HCG injection. c) 12 days after injection. d) 18 days after injection. Each symbol indicates: GA, type A spermatogonium; E-GB, early type B spermatogonium; L-GB, late type B spermatogonium; SC, spermatocyte; ST, spermatid; SZ, spermatozoon. The magnification of figures a to d is the same; bar indicates 10 μm.

type A and early type B spermatogonia were morphologically similar, with clear homogeneous nuclei containing one or two nucleoli. Each contained numerous spherical mitochondria with clear matrices; the few mitochondrial cristae observed were oriented obliquely or roughly parallel to the mitochondrial wall (Fig. 2a). Type A spermatogonia occurred singly, each cell almost completely surrounded by Sertoli cells. Early type B spermatogonia formed a cyst of two or four germ cells surrounded by Sertoli cells. Several type A spermatogonia and cysts of early type B spermatogonia formed seminal lobules that were spread out in an irregular connective tissue framework. Most of these spermatogonia were at mitotic rest, although mitotic metaphase and anaphase were observed occasionally.

One day after HCG injection, germ cells did not show any morphological changes. On day three, mitosis of type A spermatogonia and early type B

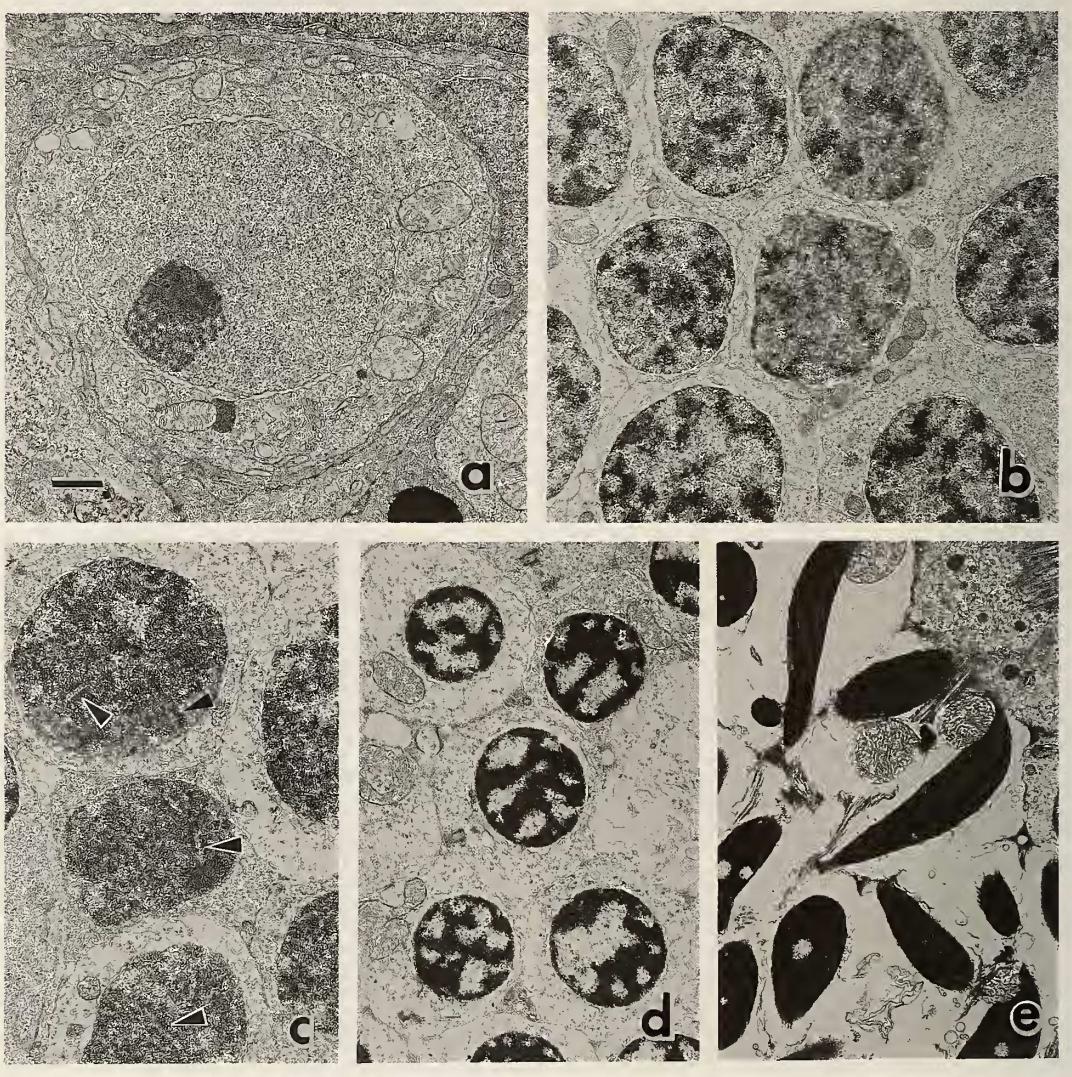

Fig. 2. Electron micrographs of germ cells in Japanese eel. a) Type A spermatogonium in the testis of an untreated eel. b) Late type B spermatogonia in the testis 3 days after HCG treatment. c) Zygotene spermatocytes with synaptonemal complexes (arrowheads) in the testis 12 days after HCG treatment. d) Spermatids 12 days after treatment. e) Spermatozoon in the tesits 18 days after treatment. The magnification of figures a to e is the same; bar indicates 1 μm.

spermatogonia was observed more frequently than on the preceding days, and late type B spermatogonia appeared (Figs. 1a, 3). Late type B spermatogonia had a dense and heterogeneous nucleus. The mitochondria had a darker matrix, and were smaller and more elongate than those of type A and early type B spermatogonia (Fig. 2b).

On day six, mitosis of spermatogonia was still observed frequently, and the percentage of late type B spermatogonia in the testis increased (Fig. 3). All spermatogonia in a single cyst occurred at the same stage of mitotic division (metaphase or anaphase). As the proliferation of spermatogonia progressed, the lobules formed by germ cells and their associated Sertoli cells were enlarged. A lumen was formed in the center of some lobules, and the connective tissue between lobules became compressed.

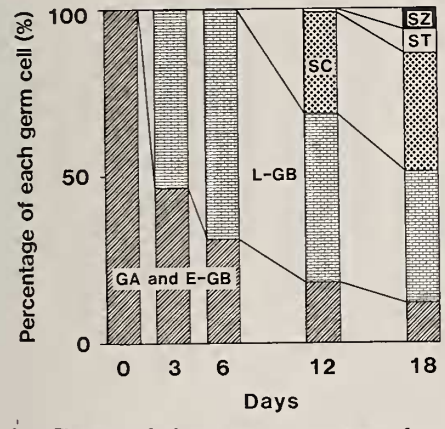

FIG. 3. Change of the mean percentage of type A spermatogonia and cysts of each germ cell type in the testis of Japanese eel after a single injection of HCG. Each symbol indicates: GA, type A spermatogonia; E-GB, early type B spermatogonia; L-GB, type B spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa.

On day twelve, leptotene and zygotene spermatocytes with synaptonemal comlexes, and spermatids having small, round and heterogeneous nuclei and a few large mitochondria with tubular cristae, were observed for the first time (Figs. 2c, d, 3). Type A spermatogonia and early type B spermatogonia were located on the edge of the lobules and a part of their Sertoli cells was attached to the basement membrane. Cysts of other germ cell typs were located haphazardly in the lobules.

On day eighteen, spermatozoa had appeared in the lumen of seminal lobules (Figs. 1d, 2e, 3). Each spermatozoon possessed a crescent-shaped nucleus. On the caudal end of the base of the nucleus, a flagellum with $9+0$ axonemal structure was attached. On the caput end of one side of the sperm nucleus, a single large and sperical mitochondrion with developed tubular cristae was attached. There was no central section connecting the flagellum and nucleus. The cysts of spermatids were located in the center of lobules or near the lumina. On day eighteen, the percentage of (cysts containing) each germ cell type was as follows: type A spermatogonia and early type B spermatogonia, $12.3\pm4.7\%$; late type B spermatogonia, $38.8\pm9.8\%$; spermatocytes, $35.3\pm13.4\%$; spermatids, $7.5\pm1.1\%$; and spermatozoa, $6.0\pm2.3\%$ (Fig. 3). There was no difference in the development of germ cells between each lobule. The connective tissue was thinly distributed among lobules.

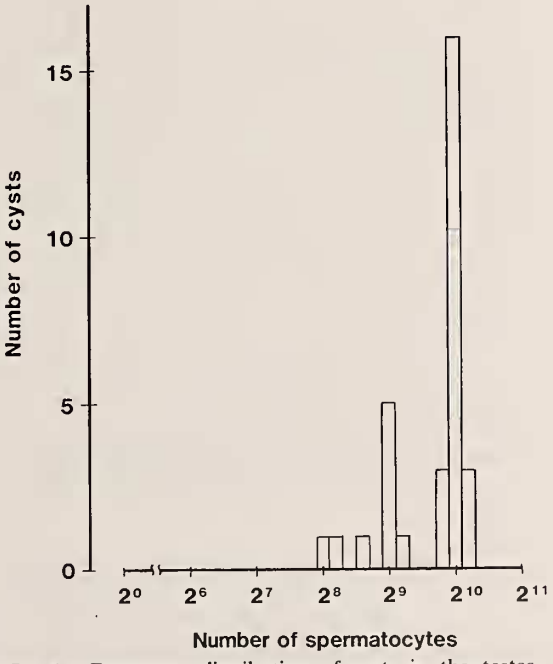

FIG. 4. Frequency distribution of cysts in the testes containing different numbers of spermatocytes, examined 18 days after HCG treatment.

The process of spermatogenesis advanced synchronously within the same cyst. Most of the spermatocyte cyst had 2^{10} cells, though a few cysts had 2^8 or 2^9 (Fig. 4).

In the saline injection group, all germ cells were type A spermatogonia and early type B spermatogonia. Germ cells of other stages were not observed throughout the experimental period.

2. *Somatic cells*

Before HCG injection, Sertoli cells enclosing spermatogonia, had irregular nuclei containing some electron dense areas, and a relatively narrow cytoplasm with poorly developed organelles (Fig. 5a). Leydig cells occurred in the interstitial tissue and had round or oval nuclei containing some electron dense areas. Their mitochondria were generally round or oval in form, but irregularly elongated in some cases. Mitochondrial cristae were usually indistinct. The endoplasmic reticulum of Leydig cells was poorly developed (Fig. 6a).

One day after HCG injection, though germ cells were morphologically unchanged, Sertoli cells and Leydig cells showed remarkable changes (Figs. 5b, 6b). In Sertoli cells, the nuclei swelled and became light and homogeneous in electron density, and the cytoplasm became filled with organelles, including Golgi complexes, smooth endoplasmic reticulum and free ribosomes. In Leydig cells, swelling of nuclei, expansion of mitochondria with tubular cristae, remarkable development of Golgi complexes and smooth endoplasmic reticulum, and an increase of free ribosomes resulted in a remarkable expansion of the cell.

From day three onwards, Sertoli and Leydig cells maintained the ultrastructural aspects found one day after injection. There were no ultrastructural differences among Sertoli cells surrounding germ cells of different spermatogenic stages, or among Leydig cells at different positions within a testis.

These changes in Sertoli and Leydig cells after HCG injection are shown quantitatively in Figures 7 and 8. The size of nuclei in Sertoli cells and that of mitochondria in Leydig cells were used as indices of cell activation, since they showed remarkable changes after HCG injection. One day

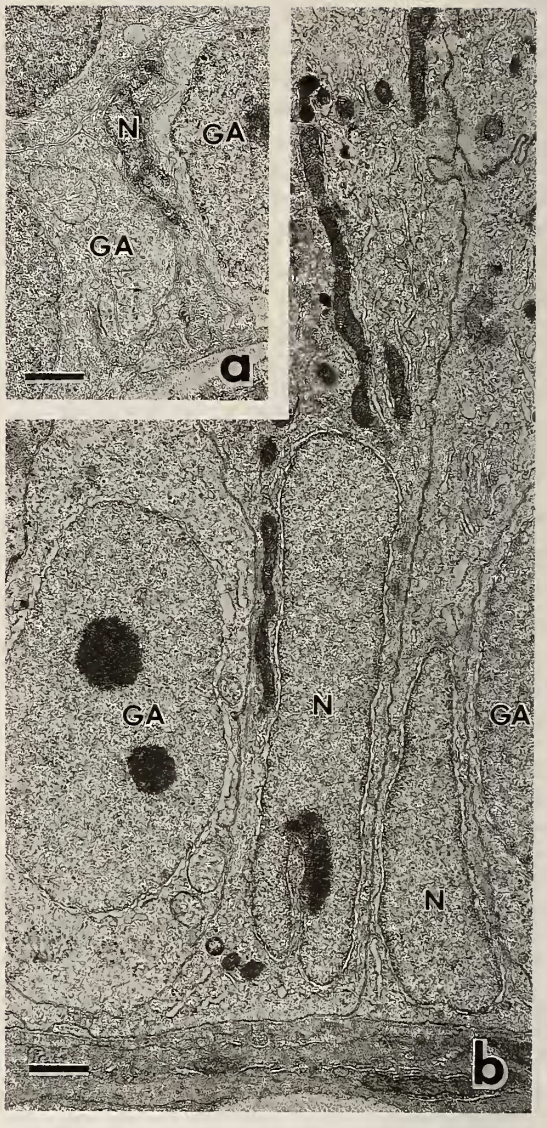

Fig. 5. Electron micrographs of Sertoli cells in cultivated Japanese eel. a) Untreated eel. b) 1 day after HCG treatment. Each symbol indicates: N, nucleus; GA, type A spermatogonium. Bars indicate 1 μm.

after HCG injection, the index of Leydig cell activation increased significantly in comparison with that found at the initial stage and in saline controls ($P<0.001$). After that, the high level of the index was maintained until the end of the experiment. The index of Sertoli cell activation also increased abruptly one day after HCG injection. Moreover, this index increased gradually

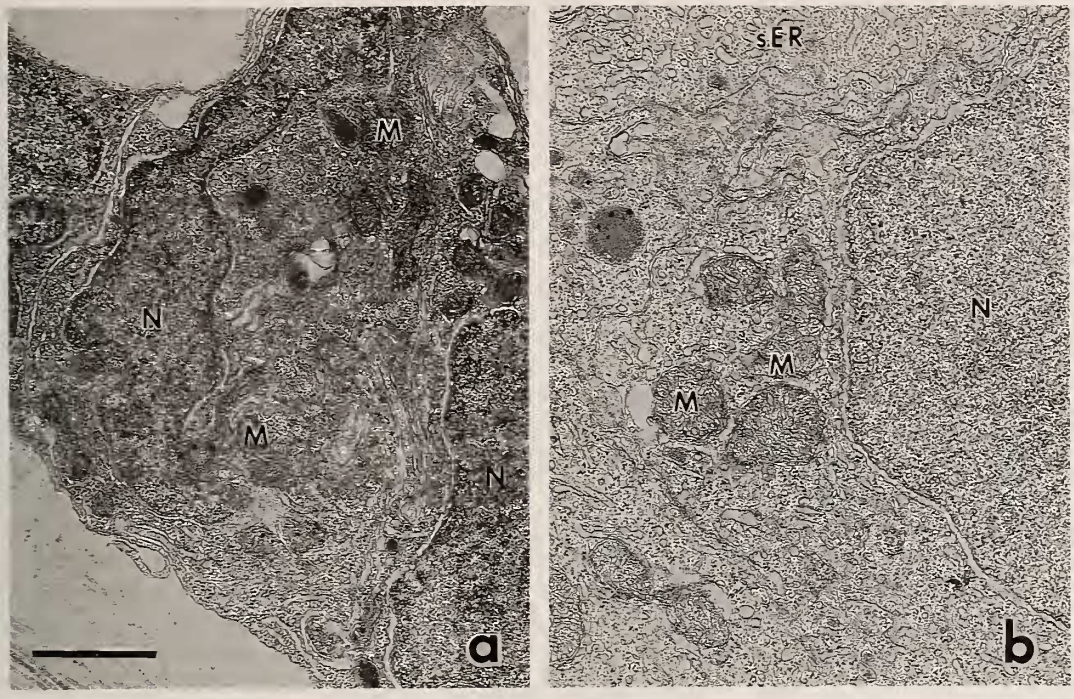

FIG. 6. Electron micrographs of Leydig cells in cultivated Japanese eel. a) Untreated eel. b) 1 day after HCG treatment. Each symbol indicates: N, nucleus; M, mitochondria; sER, smooth endoplasmic reticulum. The magnification of figures a and b is the same; bar indicates 1 μm.

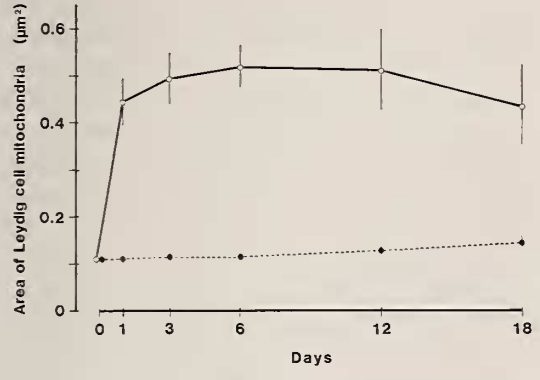

FIG. 7. Effects of a single injection of HCG on the size of Leydig cell mitochondria (solid line). Broken line indicates the saline injection group. The vertical bars represent the mean ± SEM.

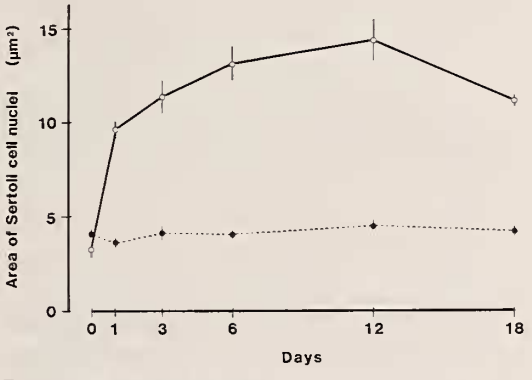

FIG. 8. Effects of a single injection of HCG on the size of Sertoli cell nuclei (solid line). Broken line indicates the saline injection group. The vertical bars represent the mean ± SEM.

until twelve days after HCG injection when some germ cells began meiosis, followed by a significant decrease on day eighteen. There was a statistically significant difference in the size of Sertoli cell nuclei between day one and day twelve of the experiment ($P<0.01$).

Time course of changes in serum steroid hormone levels after HCG injection

The time course of changes in serum 11-ketotestosterone and testosterone levels after HCG or saline injection is presented in Figure 9.

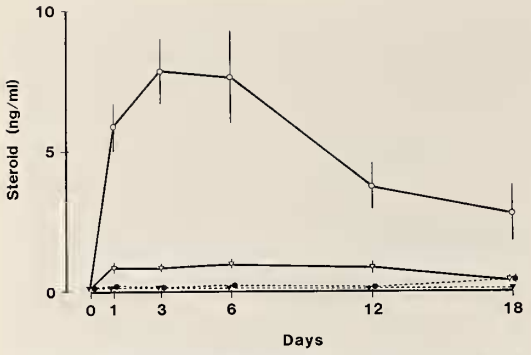

FIG. 9. Effect of a single injection of HCG on serum 11-ketotestosterone (○) and testosterone (▽). Broken lines indicate the saline injection group, 11-ketotestosterone (●) and testosterone (▼). The vertical bars represent the mean ± SEM.

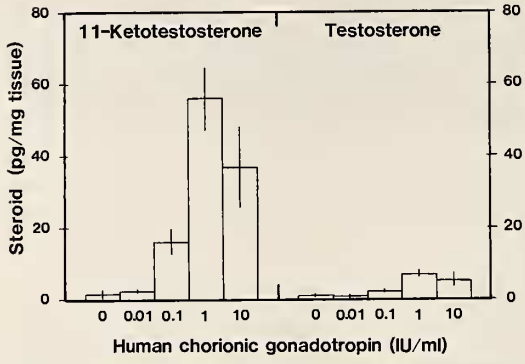

FIG. 10. Effects of HCG on 11-ketotestosterone and testosterone production by Japanese eel testicular fragments. The vertical bars represent the mean ± SEM.

Serum levels of 11-ketotestosterone and testosterone on the initial day were 0.24 ± 0.10 and 0.08 ± 0.02 ng/ml, respectively. HCG injection resulted in significant increases in 11-ketotestosterone levels as early as one day after HCG injection (5.86 ± 0.86 ng/ml). The level peaked on day three (7.83 ± 1.22 ng/ml). A similar high level was observed on day six, but the level fell significantly to 3.72 ± 0.85 ng/ml on day twelve, significantly lower than levels on day three ($P < 0.01$). Similarly, serum tetosterone levels increased on day one (0.88 ± 0.22 ng/ml). From day one onwards, serum levels of testosterone were remarkably lower than those of 11-ketotestosterone, and showed no significant changes throughout the rest of the experimental period.

The saline injection group had no significant changes in serum 11-ketotestosterone or testosterone levels throughout the experimental period.

In vitro androgen production by intact testicular fragments

Testicular fragments from uninjected eels were incubated in physiological saline solution for 18 hr in the continuous presence or absence of various concentrations of HCG (0.01, 0.1, 1, 10 IU/ml). The accumulation of 11-ketotestosterone and testosterone in the media is shown in Figure 10. The concentration of 11-ketotestosterone released into the medium was significantly stimulated ($P < 0.01$) by HCG in a dose-related manner, with the peak (56.0 ± 10.0 pg/mg tissue) at 1 IU/ml of HCG supplement. Similarly, HCG stimulated the production of testosterone by testicular fragments in a dose-related manner, however, these levels were approximately one-sixth of 11-ketotestosterone levels.

DISCUSSION

In the present study, proliferation of spermatogonia, meiosis and spermiogenesis were induced in a period of only 18 days following a single HCG injection. This confirms earlier reports that injections of either pituitary extracts or various gonadotropins induce spermatogenesis in silver European eel (*Anguilla anguilla*) [8–12] and Japanese eel [2, 13].

Spermatogonia were classified into the following three types by morphology; 1) type A spermatogonia, 2) early type B spermatogonia and 3) late type B spermatogonia. Type A and early type B spermatogonia are primitive spermatogonia which have not begun to proliferate. These two types of spermatogonia were very similar in ultrastructure, but type A spermatogonia existed as isolated cells, whereas early type B spermatogonia consisted of two or four cells within a cyst. Late type B spermatogonia resulted from the proliferation of these primitive spermatogonia and differred in ultrastructure from the earlier two types: their nucleus was denser and more heterogenous, and their mitchondria were smaller and more elongate.

The morphological differences betwen these spermatogonial generations were very similar to those described for the guppy, *Poecilia reticulata* [14].

Before HCG injection, the germ cells in testes of the Japanese eel used in the present study were type A spermatogonia and early type B spermatogonia, and there were no late type B spermatogonia or other germ cells of advanced stages in testes. These observations indicated that the spermatogenesis had not yet started in these eels. This stage of development of the eel testis was called the "early multiplication stage" in an earlier report [2].

On the third day after HCG injection, spermatogonia began proliferation, and late type B spermatogonia appeared. This stage of the testis was called the "late multiplication stage" by Yamamoto *et al.* [2]. On the sixth day, the proliferation of spermatogonia continued, but meiosis had not started yet.

On the twelfth day, spermatocytes with synaptonemal complex were first observed in the testis. This indicates that some germ cells had started meiosis between the days six and twelve. In the eel, the number of mitotic divisions of spermatogonia before entering meiosis is not yet known. In the present study, the germ cells within the same cyst were always at the same stage of development, and all spermatogonia entered metaphase or anaphase simultaneously. These results suggest that, in the eel, spermatogenesis is perfectly synchronous within the cyst, as it is in other teleosts [15]. Therefore, the number of mitotic divisions of spermatogonia before entering meiosis can be estimated from the number of primary spermatocytes in one cyst. Although the number of primary spermatocytes in some cysts was estimated at 2^8 and 2^9, most cysts contained 2^{10} primary spermatocytes. Moreover, cysts with 2^{11} or more spermatocytes were not observed. Accordingly, a type A spermatogonial stem cell may undergo 10 mitotic divisions, and occasionally may divide 8 or 9 times, before entering meiosis. In medaka (*Oryzias latipes*), it is estimated that a type a spermatogonium will yield spermatocytes following 9 to 10 mitotic divisions [16]. However, it is not clear whether this number of mitotic divisions is an inherent property of the type A spermatogonial stem cell, or is controlled by the environment, or both.

The appearance of free spermatozoa in the seminal lobules of testes by eighteen days after HCG injection indicated that some germ cells had completed meiosis and spermiogenesis. It was not clear whether these spermatozoa had normal function. Since the sperm of Japanese eel obtained after repeated injections of HCG can fertilize eggs [17, 18], it is probable that spermatozoa in the present study also have normal function. The structure and localization of spermatozoan mitochondria were different from the description given by Colak and Yamamoto [19] for spermatozoa of Japanese eel, in which small mitochondria existed in a central section of the spermatozoa. In the present study, by contrast, only one large and spherical mitochondrion existed beside the nucleus, opposite the end at which the flagellum was attached, and the central section was lacking. Other structures of spermatozoa are similar to those reported for Japanese eel [19] and European eel [20].

The first morphological changes induced in the testis by HCG treatment were seen in Leydig cells and Sertoli cells. Subsequently, the proliferation of spermatogonia, meiosis and spermiogenesis occurred. The activated morphological states of Leydig cells and Sertoli cells were maintained throughout the process of induced spermatogenesis. These results indicate that the activation of Leydig cells and Sertoli cells induced by HCG injection may trigger the proliferation of spermatogonia and meiosis, and maintain the progress of eel spermatogenesis. The activated states of these somatic cells were induced synchronously by HCG treatment regardless of their location in the testis. Furthermore, there was no difference in ultrastructure between Sertoli cells enclosing germ cells of different spermatogenic stages in HCG treated eel. The reason for these results is not clear.

Activated Leydig cells showed several features of steroid production common to teleost testes, i.e. the occurrence of mitochondria with developed tubular cristae and the development of smooth endoplasmic reticulum [12, 13, 21–23]. If there is any relationship between the induction of spermatogenesis and the activation of Leydig cells, it

would be suggested that steroid hormones produced by Leydig cells may be related to the effects on germ cells of HCG injection. It is generally assumed that, in teleosts, exogenous gonadotropin action on gonadal development is not direct, but acts through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of spermatogenesis [1]. In Japanese eel, treatment with HCG caused a dramatic increase in serum 11-ketotestosterone and testosterone levels *in vivo,* and stimulated *in vitro* production of these two androgens in intact immature testicular fragements. This indicates that the testis of Japanese eel has the ability to produce these steroids when stimulated by gonadotropins such as HCG. One or both of these steroids may act as steroidal mediators of gonadotropin-induced proliferation of spermatogonia, induction of meiosis, and spermiogenesis.

11-Ketotestosterone is a common androgen in male teleosts. This steroid is effective in causing spermiation in goldfish [24] and the expression of male secondary sex characteristics in salmonids [25] and medaka [26]. Direct evidence of a function in the proliferation of spermatogonia and induction of meiosis, however, is lacking. In the present study, serum 11-ketotesterone levels increased simultaneously with the activation of Leydig cells by HCG injection, followed by proliferation of spermatogonia and occurrence of meiosis. These results indicate a possible relationship between 11-ketotestosterone and early spermatogenesis, and that the source of 11-ketotestosterone may be the HCG activated Leydig cells.

Testosterone is an intermediate androgen product in the synthesis of 11-ketotestosterone [27], and its androgenic activity seems to be lower than that of 11-ketotestosterone in medaka [26]. Although, in the present study, testosterone concentrations in the serum and incubation medium were increased by HCG stimulation, its concentration was much lower than 11-ketotestosterone. These results suggest that the major function of testosterone may be as a precursor to 11-ketotestosterone.

Although morphological activation of Sertoli cells occurred following HCG injection, the significance of this activation is not clear. The most important role of Sertoli cells is thought to be the secretion of steroid or protein mediators of spermatogenesis [15, 28, 29]. In the present study, Sertoli cells activated by HCG treatment did not show the typical ultrastructure of steroid producing cells. This suggests that the action of Sertoli cells on spermatogenesis may not be mediated by steroids. In mammals, it is supposed that the factor directly controlling spermatogenesis (mainly, mitosis of spermatogonia) is not testosterone and/or gonadotropin, but a protein, seminiferous growth factor (SGF) that is produced by Sertoli cells [30]. Similar morphological changes of Sertoli cells could be induced by the incubation of testis fragments of Japanese eel with 11-ketotestosterone *in vitro* (unpublished data). This suggests that the morphological activation of Sertoli cells is controlled by 11-ketotestosterone. The relationship between the activation of Sertoli cells and the induction of spermatogenesis is one of the most important problems in resolving the mechanisms of spermatogenesis.

In conclusion, the results suggest the following senario, explaining induction of eel spermatogenesis by gonadotropin (HCG): HCG injected in the eel stimulates Leydig and/or Sertoli cells, which as a result, produce androgens (especially 11-ketotesterone). These androgens are related to the induction of completed spermatogenesis from premitotic spermatogonia to spermatozoa, acting either directly or through the Sertoli cells. Thus, the Japanese eel provides an excellent system for analysis of the control mechanisms of spermatogenesis.

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